Original Article

Deterioration of wound healing and intense suppression of MMP-9 mRNA expression after short-term administration of different topical glucocorticoids or NSAIDs in an avian model of corneal lesions

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Abstract

Background: Corneal lesions are considered among important ophthalmic conditions in avian patients. Short-term outcome of using anti-inflammatory agents in corneal lesions of birds are not well-described. **Aims:** The study evaluates effects of different anti-inflammatory agents on healing of alkali burn-induced corneal lesions in layer hens as an avian model. **Methods:** Adult layers were randomly allocated into 7 groups (n=15) as follows: 1. Negative (normal) control (NC), and 2. Positive control (PC) with an experimentally induced-corneal lesion, 3-7. Birds with corneal lesions that were treated with dexamethasone, fluorometholone, prednisolone, ketorolac, or diclofenac eye-drops every 6 hours (QID) for 5 consecutive days. **Results:** At the end of the experiment, proper healing was observed in PC group based on lesion area, while treated groups showed statistically larger lesion sizes as compared to PC birds ($P<0.05$). Although no significant difference was observed among groups, birds treated with ketorolac, diclofenac or fluorometholone had higher histopathological scores for most of the assayed parameters than other groups. Levels of tumor necrosis factor-α (TNF-α) and vascular endothelial growth factor (VEGF) in corneal tissue of different groups were statistically the same. The mRNA expression of matrix metalloproteinase-9 (MMP-9) was increased 2.5 folds in PC group as compared to NC birds. However, birds treated with anti-inflammatory agents showed no detectable expression of MMP-9 mRNA. **Conclusion:** Five days of topical administration of non-steroidal anti-inflammatory agents (NSAIDs) or glucocorticoids (GCs) is associated with suppression of MMP-9 mRNA expression in corneal tissue and detrimental effects on wound healing in layers with alkali burn-induced corneal ulcers.

Key words: Anti-inflammatory agents, Bird, Cornea, Healing, Matrix metalloproteinase-9

Introduction

Corneal lesions including abrasions and ulcers are among ophthalmic conditions that are diagnosed in avian patients referred to veterinary clinics. Despite having eyelids, filoplumes, and nictitating membranes, different factors including mechanical or traumatic insults in hunting or during reproductive activities can lead to severe corneal lesions in birds. Moreover, ulcerative keratitis secondary to infections is important in avian species. Like mammals, fluorescein staining is routinely used for the diagnosis of corneal ulcers in avian species (Williams, 1994; Carvalho *et al*., 2018).

Although corneal ulcers usually heal rapidly, management of pain and inflammation as well as prevention of infection-induced damage are important steps to avoid chronic inflammation that may lead to pigmentation and neovascularization of the cornea. Neovascularization is a serious sequela of corneal lesions and can result in complete blindness. Corneal alkali burn-induced injuries have been properly used as models for evaluation of agents with possible effects on corneal neovascularization (Anderson *et al*., 2014). It has been clearly demonstrated that tumor necrosis factor-α (TNF-α), as an outstanding pro-inflammatory cytokine, can promote neovascularization of cornea by different mechanisms (Ferrari *et al*., 2015). Vascular endothelial growth factor (VEGF) also has a remarkable role in the development of corneal neovascularization after different insults. Current therapies for corneal neovascularization mostly rely on topical glucocorticoids (GCs) and non-steroidal anti-inflammatory agents (NSAIDs) (Stevenson *et al*., 2012).

Matrix metalloproteinases (MMPs) are endopeptidases which are produced by different cell types and have a role in diverse physiological and pathological processes such as healing. Corneal epithelial cells produce matrix metalloproteinase-2 (MMP-2) and MMP-9 as their major matrix-degrading enzymes. The latter is the most important enzyme that degrades

epithelial basement membrane components. It has been demonstrated that TNF-α upregulates MMP-9 expression at both mRNA and protein level in primary human corneal epithelial (HCE) cell cultures in a dose-dependent manner (Li *et al*., 2001). Moreover, activated macrophages can upregulate VEGF and MMP-9 expression in rat corneal tissue (Li *et al*., 2012).

Unfortunately, short-term management protocols and possible drug options for the control of pain and inflammation in corneal lesions of avian species are not well-described. Most data are derived from human medicine where the use of NSAIDs in corneal lesions has shown some inconsistencies. Some reports declare that they can be effectively used in the short-term management of pain and inflammation associated with corneal abrasions without complicating wound healing (Calder *et al*., 2005; Smith and Goldman, 2012; Thiel *et al*., 2017), while other studies performed on animal models (Iwamoto *et al*., 2017) or corneal cell cultures report deleterious effects of NSAIDs on corneal lesions (Li *et al*., 2021).

Although GCs are among the most widely prescribed pharmacological agents in ophthalmic conditions, their use in corneal lesions is also controversial. Where a prospective, randomized, placebo-controlled trial on steroids for corneal ulcers (SCUT) shows that topical corticosteroids are safe in patients with bacterial corneal ulcers (Srinivasan *et al*., 2014), a study in rabbits with corneal ulcers reported that topical dexamethasone eye-drops twice a day for 5 days, slows wound healing (Araki-Sasaki, 2016). It is routinely declared that topical GCs can potentiate corneal ulceration (Stanley, 2008).

The lack of information on the possibility of using steroidal and NSAIDs in short-term management of corneal lesions in avian species, prompted us to evaluate the effects of routinely used three different topical GCs, and two NSAIDs on the healing outcome of alkali burn-induced corneal lesions in layer hens as a model for other avian species. Lesion size, histopathological features, levels of TNF-α, VEGF, and mRNA expression of MMP-9 in corneal tissue are assayed.

Materials and Methods

Experimental design

The study was performed using a parallel-controlled experimental design. One hundred and five commercial Lohman-LSL adult (70 weeks of age) layer hens with a body weight (BW) of 1463 ± 183 g, were randomly allocated into 7 equal groups (n=15 each). After a week of adaptation, birds were treated as follows: 1. Negative (normal) control (NC) group (birds with normal cornea), 2. Positive control (PC) group, corneal lesion was induced by NaOH alkali burn and these birds received no special treatment (only artificial tear-drops with the same frequency as treated groups), and 3-7. Treatment groups, birds with alkali burn-induced corneal lesion that were treated with dexamethasone 0.1% (dex) (Dexon®, Sina Darou, Iran), fluorometholone 0.1% (flu) (Fluocort®, Sina Darou, Iran), prednisolone acetate 0.1% (pred) (Precord®, Sina Darou, Iran) (GC groups), ketorolac tromethamine 0.5% (keto) (Sinarolac®, Sina Darou, Iran), and diclofenac sodium 0.1% (dic) (Voldic®, Raha Pharma Co., Iran) (NSAID groups) eye-drops every 6 hours (QID) one drop/eye for 5 consecutive days. The dosage regimen was based on drug labels as described by the manufacturer. All drugs were formulated for human use.

Birds were kept in separate cages and had free access to drinking water and standard feed formulated based on their needs as described by the rearing manual.

All procedures used in the study are in accordance with institutional ethical guidelines which are based on the Directive 2010/63/EU on the protection of animals used for scientific purposes.

Induction of corneal alkali burn lesion

The method used for induction of the corneal lesion was based on previous reports in mice (Bai *et al*., 2016) and rabbits (Griffith *et al*., 2018). Since no study was available for the induction of alkali burn corneal lesions in birds, a pilot study was performed using different concentrations of NaOH as well as different contact times. Finally, the lesion was induced by filter papers with 5 mm diameters that were soaked in 1 M NaOH and were placed for 30 s on the center of the right cornea. After lesion induction, the cornea was completely washed with normal saline for 1 min. Before lesion induction, birds were generally anesthetized with intramuscular injection of ketamine (40 mg/kg) and midazolam (2 mg/kg) in pectoral muscles. To reduce post induction pain, tetracaine 0.5% eye drop was instilled once before placing the disks on the corneal surface. Fluorescein staining was performed on days 1 and 6 after lesion induction. Then, photographs were made and area of the stained lesion was calculated using Zeiss AxioVision LE® software.

On day 6, corneal samples were obtained from all birds after euthanasia by cervical dislocation. Samples from each group were randomly divided into 3 equal subgroups for histopathological and biochemical (TNF-α and VEGF) assays as well as determination of MMP-9 mRNA expression.

Histopathological evaluation

Corneal samples from each group were fixed in 10% formalin and after routine procedures, 5 µm-thick perpendicular sections were prepared and stained with haematoxylin and eosin (H&E) method. All samples were evaluated under a light microscope and then were semi-quantitatively scored for lesions in epithelium including epithelial erosion, vacuolation and necrosis (0, 0.5, 1, 2, and 3 for normal, very slight, slight, moderate, and severe lesions), pyknotic nuclei in stroma (0, 1, and 2 for normal, slight, and moderate lesions) as well as presence of the stromal fiber disorders or endothelial necrosis based on a scoring system provided by OECD Annex II (2016).

Biochemical assays

Corneal samples were kept at -70°C until use. Samples were freeze-thawed two times in liquid nitrogen and then homogenized in phosphate-buffered saline (PBS) (pH = 7.4), centrifuged at 650 g for 20 min and supernatants were carefully collected. Chicken TNF-α, and chicken VEGF-A sandwich enzyme linked immunosorbent assay (ELISA) kits, both produced by Bioassay Technology Laboratory, China, were used for quantitative assay of TNF-α and VEGF in corneal tissue. The intra-assay and inter-assay coefficients of variation (CVs) of both kits were <8% and <10%, respectively. The procedure was performed as described by the manufacturer.

Quantitative real-time polymerase chain reaction (PCR) for quantitation of MMP-9 mRNA expression

Total RNA was extracted from corneal tissue using SinaPure™ RNA kit (SINACLON, Iran) as per the manufacturer's instructions. The purity of RNA was assessed by calculating OD260/OD280 using a Nano drop spectrophotometer (NanoDrop, Thermo Scientific, USA). Normalization and DNase treatment were performed using DNaseI, RNase-free (500 U) (SINACLON, Iran) according to the manufacturer's instruction. The cDNA was then prepared from total RNA using Sinaclon First Strand cDNA Synthesis kit (SINACLON, Iran) as per the manufacturer's instructions in a total volume of 20 µL (3 µL of treated RNA was used). The prepared cDNA was stored at -20°C for downstream applications. The expression of MMP-9 mRNA was carried out by using Roche lightCycler 480 (Roche, Germany) real-time PCR system. A set of primer sequences (Table 1) of chicken *MMP-9* gene were used that was previously described by Nyati *et al*. (2017). The reaction was performed in a 20 µL volume containing 3 µL cDNA, 10 µL 2x SYBR GreenMaster Low ROX quantitative PCR (qPCR) master mix (JenaBioscience, Germany), 3 µL of forward (600 nM), 1.5 µL of reverse (300 nM) MMP-9 specific primers, and 2.5 µL of nuclease free H_2O. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was utilized as the reference gene. The reaction mixture was subjected to initial denaturation at 95°C for 15 min followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 61°C for 45 s, and extension at 72°C for 30 s. All experiments were carried out in triplicate. Optical data were analyzed using LightCycler® 96 SW 1.1 data analysis software. The PCR threshold cycle (Ct) values were normalized to the expression levels of the internal housekeeping gene, GAPDH, and expressed as fold change. Interpretation of the results and determination of relative expression levels of MMP-9 mRNA were performed using the Pfaffl method (Pfaffl, 2001).

Statistical analysis

Data related to histopathological scorings were analyzed by Kruskal-Wallis test followed by Dunn's multiple comparisons test. Fisher's exact test was performed for endothelial necrosis and stromal fiber disorder data. Other data were analyzed by one-way ANOVA method followed by Tukey's multiple comparison test. $P<0.05$ was considered as the level for significant difference in all comparisons. Data analysis was performed by GraphPad Prism 6 software.

Table 1: Primer sequences for real-time qPCR (Nyati *et al*., 2017)

Gene	Primer sequences (5´-3´)
GAPDH	F: TGCCATCACAGCCACACAGAAG
	R: ACTTTCCCACAGCCTTAGCAG
MMP-9	F: GCTACCCCCGTGACACTGAT
	R: GAAGCTGTCCCGGCAGAAG

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase (Housekeeping gene), *MMP-9*: Matrix metalloproteinase-9, F: Forward, and R: Reverse

Results

Corneal lesion size

Figure 1 shows typical photographs of fluorescein-stained corneal lesions of birds in different groups. As previously stated, corneal lesion area was determined on days 1 and 6 after induction of lesion. On day 1, the lesion area was statistically the same in all groups with corneal alkali burn and showed significant difference with NC birds ($P<0.0001$) (Fig. 2A). On day 6, the lesion area of birds in PC group was reduced considerably and no significant difference was observed between NC and PC birds ($P>0.05$). Groups receiving topical GCs or NSAIDs showed statistically larger lesion sizes as compared to PC birds ($P<0.05$ for GCs and $P<0.0001$ for NSAIDs). Although no significant difference was observed among treated groups, birds that received NSAIDs had slightly larger corneal lesions compared to GC-treated groups (Fig. 2B).

Histopathological features

Figure 3 shows photomicrographs of corneal tissue from birds in different groups at the end of experiment. Birds in NC group showed no lesion, while infiltration of

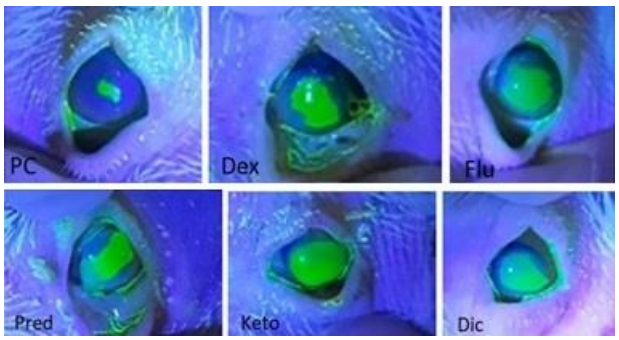

Fig. 1: Typical photographs of fluorescein stained corneal lesions of birds in different groups on day 6. PC: Positive control with corneal lesions that received no treatment, Dex, Flu, Pred, Keto, and Dic: Birds that received dexamethasone, fluorometholone, prednisolone, ketorolac, and diclofenac eye-drops, respectively

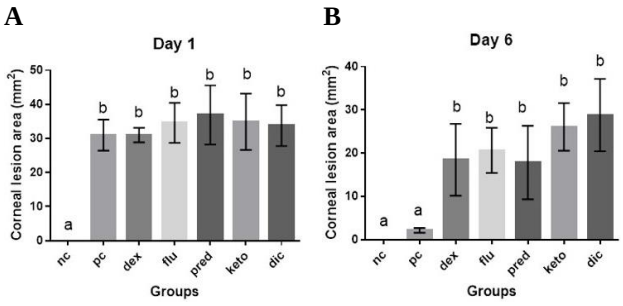

Fig. 2: Corneal lesion area (mean±SD) in birds of different groups on days 1 (**A**) and 6 (**B**) after alkali burn induction. nc: Negative (normal) control with normal cornea, pc: Positive control with corneal lesions that received no treatment, dex, flu, pred, keto, and dic: Birds that received dexamethasone, fluorometholone, prednisolone, ketorolac, and diclofenac eye-drops, respectively. Columns with different letters are significantly different at P<0.05

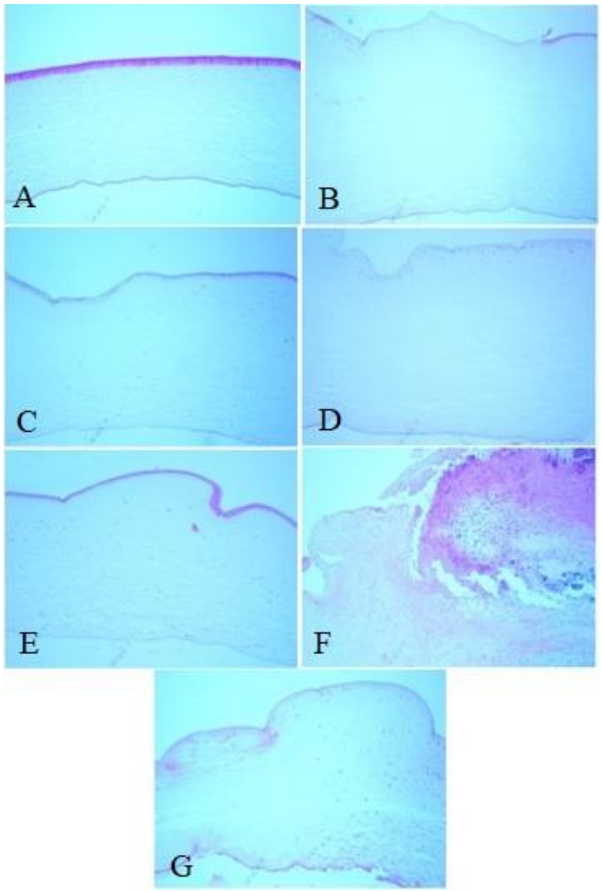

Fig. 3: Photomicrographs of corneal tissue from birds in different groups (H&E, ×100) on day 6. **A**: Negative (normal) control (NC) group with normal cornea, **B**: Positive control (PC) group, necrosis of epithelial layer and stroma as well as necrosis of endothelium, **C**: Dexamethasone-treated group, moderate vacuolation and slight epithelial erosion, **D**: Fluorometholone-treated group, severe necrosis of epithelial layer and stroma as well as necrosis of endothelium, **E**: Prednisolone-treated group, very slight vacuolation and epithelial erosion, **F**: Ketorolac-treated group, severe necrosis of epithelial layer and stroma with large number of heterophils along with bacterial colonization, and **G**: Diclofenac-treated group, severe necrosis of epithelial layer and stroma with few heterophils and necrosis of endothelium

few heterophils was observed in some birds of PC group. Among birds treated with GCs, the severity of inflammation and the infiltration of heterophils were more prominent in fluorometholone group followed by prednisolone; while birds in dexamethasone group had the least inflammatory changes. Birds treated with ketorolac had the worst outcome with severe inflammation and infiltration of many heterophils with one bird showing severe corneal tissue necrosis and colonization of bacteria. One bird in diclofenac-treated group also showed severe heterophils infiltration and necrosis.

Data related to semi-quantitative histopathological scoring of corneal lesions in different groups are summarized in Table 2. Although no significant difference was observed among groups, birds treated with ketorolac and diclofenac as well as fluorometholone had higher median scores for most of the assayed parameters than other groups.

Table 3 shows the percentage of samples with endothelial necrosis and stromal fiber disorders in different groups. No significant difference was observed in these parameters among birds treated with GCs or NSAIDs or birds of PC group (P>0.05).

Levels of TNF-α and VEGF in corneal tissue

Figure 4A shows that levels of TNF-α in corneal tissue of different groups were statistically the same (P>0.05).

While there was no significant difference among groups treated with GCs or NSAIDs with PC group, birds in dexamethasone group showed significantly higher levels of VEGF as compared to birds in fluorometholone and diclofenac groups (P<0.05 for both comparisons) (Fig. 4B).

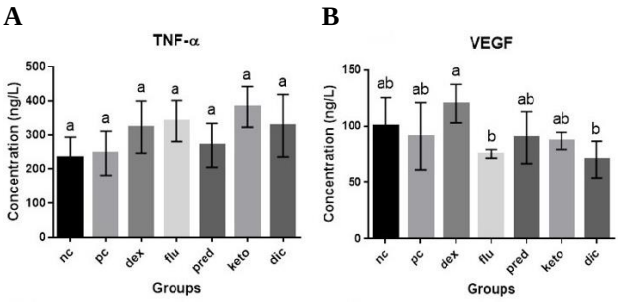

Fig. 4: levels (mean±SD) of TNF-α and VEGF in corneal tissue of birds in different groups. nc: Negative (normal) control with normal cornea, pc: Positive control with corneal lesions that received no treatment, dex, flu, pred, keto, and dic: Birds that received dexamethasone, fluorometholone, prednisolone, ketorolac, and diclofenac eye-drops, respectively. Columns with different letters are significantly different at P<0.05

MMP-9 mRNA expression

Level of MMP-9 mRNA expression showed a 2.5-fold increase in corneal tissue of PC birds as compared to NC group. However, in birds that were treated with NSAIDs or GCs no expression of MMP-9 mRNA was

Table 2: Median (range) of histopathological parameters in different groups on day 6

Histopathological parameters	NC	PC	dex	flu	pred	keto	dic
Epithelial erosion	0 (0)	1 (0.5-3)	1 (0.5-2)	3 (0.5-3)*	1.75 (0.5-3)	3 (0.5-3)**	2 (0.5-3)
Epithelial vacuolation	0 (0)	0.5 (0-1)	1 (0.5-2)	0 (0-1)	0.25 (0-0.5)	0 (0-0.5)	1 (0-2)
Epithelial necrosis	0 (0)	1 (0-3)	0 (0)	3 (0-3)	1.5 (0.3)	3 (0-3)	3 (0-3)
Pyknotic nuclei in stroma	0 (0)	1 (0-2)	1 (0-2)	2 (0-2)	1 (0-2)	2 (0-2)	2 (1-2)*

*, ** Signs show significant difference with NC group at P<0.05 and P<0.01, respectively. No significant difference was observed among other groups (P>0.05). NC: Negative (normal) control with normal cornea, PC: Positive control with corneal lesions that received no treatment, dex, flu, pred, keto, and dic: Birds that received dexamethasone, fluorometholone, prednisolone, ketorolac, and diclofenac eye-drops, respectively

Table 3: Percentage of samples with endothelial necrosis and stromal fiber disorders in different groups on day 6

Histopathological parameters	NC	PC	dex	flu	pred	keto	dic
Endothelial necrosis	0%	100%**	75%*	80%*	75%*	80%*	60%*
Stromal fibers disorders	0%	0%	0%	20%	0%	20%	0%

*, ** Signs show significant difference with NC group at P<0.05 and P<0.01, respectively. No significant difference was observed among other groups (P>0.05). NC: Negative (normal) control with normal cornea, PC: Positive control with corneal lesions that received no treatment, dex, flu, pred, keto, and dic: Birds that received dexamethasone, fluorometholone, prednisolone, ketorolac, and diclofenac eye-drops, respectively

detected in real-time PCR assay.

Discussion

Our study investigated the effects of short-term administration of five different GC and NSAID eye-drops on alkali burn corneal injury in layer hens as a model for avian species.

Based on our observations, GCs and NSAIDs (with slightly more intensity) adversely affect wound healing in birds' corneas with alkali burn injury as demonstrated by evolution of large wounds with more severe histopathological changes. This is in stark contrast with some previous clinical reports in humans that support short-term administration of these agents for pain and inflammation management of corneal injuries without appreciable deterioration of wound healing (Calder *et al*., 2005; Smith and Goldman, 2012; Srinivasan *et al*., 2014; Thiel *et al*., 2017).

Although the avian cornea is histologically similar to mammals with five layers, cornea of most bird species is thinner than mammals (Carvalho *et al*., 2018). Therefore, it seems that the penetration of topical agents to corneal structures of birds may be more prominent than their mammalian counterparts. The relatively short dosing intervals may also play a role in this regard.

Different mechanisms are proposed for the deleterious effects of NSAIDs or GCs on corneal lesions. For instance, in a study by Iwamoto *et al*. (2017); short-term diclofenac eye-drop QID delayed wound healing in a mouse model of surgically-induced corneal ulcer by reducing production of 12-hydroxyheptadecatrienoic acid, as a ligand for leukotriene B4 receptor 2. Diclofenac has also triggered apoptosis in HCE cells (Li *et al*., 2001). Induction of apoptosis in human corneal cells (Ryu *et al*., 2017) as well as attenuation of cell migration (Kadmiel *et al*., 2016) have also been reported due to corticosteroids.

Chemical burns result in a severe increase in VEGF expression in affected corneal tissue of humans (Philipp *et al*., 2000). On the other hand, expression of TNF-α as one of the most potent inflammatory cytokines is upregulated 24 h after alkali burn injury in mice (Cade *et al*., 2014). In contrast, we did not find a significant difference in VEGF or TNF-α levels between NC and PC groups on day 6 of the experiment. This observation may be related to the time of sampling and/or proper healing with relatively low histopathological lesions in PC group.

Interestingly, despite the presence of more severe lesions and larger wounds in GC and NSAID-treated groups, we did not find a significant difference in VEGF levels of corneal tissues from these birds as compared to that of PC group. Administration of NSAIDs has been associated with a significant decrease of VEGF levels in injured rat corneas (Castro *et al*., 2004) and dexamethasone has downregulated VEGF expression in immortalized HCE cells (Kadmiel *et al*., 2016). Therefore, the reason that we did not find a significant change in corneal VEGF level of treated vs. non-treated birds, can be related to the suppressing effects of anti-inflammatory agents on VEGF levels, despite the presence of large wounds.

As aforementioned, birds treated with NSAIDs especially ketorolac as well as birds that received fluorometholone had the worst outcome with severe inflammation in histopathological evaluation. However, we observed only a slight increase in TNF-α levels of birds in these groups compared to PC birds. In the cornea, TNF-α is expressed by the epithelium, stroma, and endothelium and inflammation can increase its expression by these three layers. Inflammatory cells, primarily activated macrophages and T lymphocytes, can also produce this cytokine (Ferrari *et al*., 2015). In histopathological evaluation, we observed severe lesions in the corneal epithelium, stroma, and endothelium of NSAID-treated birds as well as birds in the fluorometholone group. Inflammatory cells in corneal

slides at the time of sampling were mostly heterophils. These observations can describe the insignificant increase in TNF-α level of corneal tissue in birds treated with NSAIDs or fluorometholone despite obvious inflammation. On the other hand, it has been clearly demonstrated that GCs can suppress TNF-α synthesis by activated inflammatory cells, *in vivo* (Steer *et al*., 2000). This effect may also be important in explaining statistically the same levels of TNF-α which was observed among GC-treated and NC or PC birds.

In 2016, Gao *et al*. reported that alkali burn corneal injury in rabbits is associated with a significant increase in MMP-9 level of aqueous humor (Gao *et al*., 2016). Despite proper healing of the corneal ulcer, we observed a 2.5-fold increase in mRNA expression of MMP-9 in corneal tissue of PC birds as compared to NC group. Although excessive expression of MMPs can be detrimental, these enzymes have a decisive role in all stages of the corneal healing process. They degrade the extracellular matrix and therefore play an important role in wound healing (Singh *et al*., 2012). Matrix metalloproteinase-9 contributes to epithelial repair and remodeling of the stroma and also promotes migration of basal epithelial cells. It has a role in remodeling the subepithelial basement membrane region (Kaya *et al*., 2021). These roles can describe the reason behind the increased mRNA expression of MMP-9 which was observed in PC group of the current study.

It has been well established that the release of cytokines in corneal injuries can trigger up regulation of several MMPs by epithelial cells. Granules of recruited neutrophils are also a source of preformed MMP-9 enzyme (Mulholland *et al*., 2005). Interestingly, despite the presence of heterophils in corneal tissue of birds with alkali burn treated with GCs or NSAIDs, we did not detect MMP-9 mRNA in corneal tissue of these groups. This observation could be related to severe destruction of epithelial cells as a major source for enzyme production. Suppressing effects of GCs on MMP-9 expression may also be important. Accordingly, De Paiva *et al*. (2006) clearly showed that methylprednisolone suppresses MMP-9 expression in corneal epithelium of mice with experimental dry eye (De Paiva *et al*., 2006). The suppressive effect of dexamethasone on MMP-9 expression in corneal lysates has also been reported in mice with an ocular alkali burn along with desiccating stress (Bian *et al*., 2016). Limited knowledge is available on the effect of NSAIDs on MMP-9 expression in corneal injuries. In a study by Reviglio *et al*., 2003, administration of ketorolac or diclofenac eye-drops was not associated with a significant change in enzymograms of rat corneas with debrided epithelium as compared to animals that received artificial tears. This inconsistency may be related to different animal models or other determining factors.

In conclusion, 5 days of topical administration of NSAIDs or GCs is associated with suppression of MMP-9 mRNA expression in corneal tissue and detrimental effects on corneal wound healing in layers with alkali burn-induced corneal ulcers.

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Conflict of interest

Authors declare no conflict of interests.

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